mutants, sites previously shown to be involved in BK Ca^{2+}-sensitivity and located closely to D369, also affected channel-PIP_2 affinity. Our results show that the IC_{50} values for neomycin effects on both D362G and D367G were largely decreased (~100-fold), indicating that the mutations weakened channel-PIP_2 interaction. In contrast, the D369G mutation increased the IC_{50} for neomycin in a voltage-dependent manner, suggesting an enhanced channel-PIP_2 interaction. Taken together, these results suggested that mutation of the negatively charged residues D362 and D367, which lowers Ca^{2+} affinity, also decreases channel-PIP_2 affinity, while the mutant D369G, which increases Ca^{2+} affinity also enhanced channel-PIP_2 interaction. Thus Ca^{2+} and PIP_2 affinities are interrelated down to the single site interaction level.

3155-Pos Board B260
Structure-Function Studies of the Large Conductance Voltage-and Calcium-Activated Potassium Channel BETA1 Auxiliary Subunit
Bin Wang, Aleksandra Gruslova, Iurii Semenov, Robert Brenner.
BK channels are large conductance, voltage- and calcium-activated potassium channels. In smooth muscle, these channels maintain a polarized negative membrane potential and therefore deactivate calcium influx through voltage-gated calcium channels and reduce contraction. The smooth muscle-specific auxiliary β1 subunit confers increased BK opening and therefore plays an essential role in limiting smooth muscle contraction. β1 has at least two opposing gating effects: reducing intrinsic opening and stabilizing voltage-sensor activation. The goal of this research was to identify residues and structural domains that mediate these gating effects. Here, we performed an Alanine-scanning mutagenesis of β1 residues identical between β1, β2 and β4 based on the assumption that functionally important residues are likely to be conserved among family members. Effects of the Alanine mutants were categorized based on steadystate and kinetic effects on gating at 60, 4 micromolar and nominal 0 calcium. We found that 17 mutants display moderate or no effects at all calcium. 10 Class I mutants reduce conductance-voltage shifts at both high and nominal 0 calcium. Interestingly, all Class I residues are non-polar, and reside in the extracellular loop. This suggests that extracellular key residues may promote opening by providing a non-polar environment to the activated voltage senor. The only Class II mutant, G171A, abolished β1's ability in reducing opening in the absence of calcium. This suggests that G171 plays a critical role in reducing intrinsic opening. Finally, the mutagenesis data suggests that 2 evolutionarily conserved Cysteine pairs and their disulfides may play an important role in the extracellular β subunit structure. Combining single and double mutations, we found that the two amino acid pairs C53-C135 and C76-C103, may serve to form disulfide bonds that bring key extracellular Class I residues in close approximation.

3156-Pos Board B261
Investigation of BK Channel Gating using Mallotoxin
Janos Almassy, Ted Begenisich.
While large-conductance, BK K^+ channels are activated by both voltage and by Ca^{2+} in the microM range, the channels can be activated by voltage even in the absence of Ca^{2+}. BK channel β-subunits, several chemicals, and mutations can shift voltage activation toward more hyperpolarized potentials but in a rather complex manner. In contrast the accessory protein leucine-rich repeat containing protein 26 (LRRC26) and mallotoxin each appears to produce a simple shift. We have investigated the functional basis for the gating shift produced by mallotoxin in the absence and presence of the LRRC26 protein in the context of the Horrigan-Aldrich (HA) model. In the absence of intracellular Ca^{2+}, we found that, in addition to a hyperpolarizing shift of BK activation, mallotoxin produced a large, hyperpolarizing shift of channel activation kinetics. This result suggests that a major action of mallotoxin is to sensitize the BK channel voltage sensors. We also found that the degree of gating shift of mallotoxin was significantly decreased when co-expressed with the LRRC26 protein or in native parotid acinar cells that endogenously express this accessory protein. These results suggest that there is limit to the degree of sensitization of the BK channel voltage sensors.

3157-Pos Board B262
Cholesterol Regulates the Basal Functions and Ethanol Sensitivity of Large Conductance, Ca^{2+}-Sensitive K+ channel through Specific Cholesterol-Protein Interaction
Chunbo Yuan, Steven N. Treistman, Douglas F. Covey, Maohui Chen, Linda J. Johnston.
Membrane cholesterol plays an important role in regulation of a variety of ion channels and in maintaining the normal functions of cell membranes. The mechanism underlying this influence on ion channels, however, remains poorly understood. Cholesterol can act on ion channel proteins through either: 1) direct interaction with the protein, or 2) indirect effects on the biophysical properties of lipid bilayer. To differentiate between these alternatives, we used a synthesized enantiomer of cholesterol (ent-CHS). Enantiomeric forms of cholesterol have been used in the study of cholesterol-protein interaction, based upon the assumption that the effect on the lipid bilayer will be identical to that of natural cholesterol (nat-CHS), steric interaction directly with the protein will be abolished. We employed planar bilayer recording techniques to study the interaction of nat-CHS and ent-CHS with the large conductance, calcium-sensitive potassium channel (BK) in lipid bilayers of POPE/POPS (3/1, weight) and DOPE/SPM (3/2, weight), and examined how the presence of nat-CHS and ent-CHS affected the basal function and ethanol sensitivity of the BK channel. We found that ent-CHS increased BK channel conductance in both lipid bilayers similarly to that of nat-CHS. However, they are strikingly different in their effects on BK channel gating and ethanol sensitivity. In the POPE/POPS bilayer, nat-CHS dramatically reduced the open probability (Po) while ent-CHS did not. Both ent-CHS and nat-CHS reduced the ethanol sensitivity of the BK channel, but ent-CHS did so to a lesser extent. In the DOPE/SPM bilayer, nat-CHS dramatically changed the ethanol response of BK, depending on cholesterol concentration in the membrane. However, ent-CHS had little effect on ethanol sensitivity. We conclude that membrane cholesterol has a specific interaction with the BK channel that can directly influence ethanol's actions.

3158-Pos Board B263
The Slo1 C-Tail Domain Confers Cholesterol-Sensitivity to Arterial Smooth Muscle BK Channels
Aditya K. Singh, Anna N. Bukiya, Alejandro M. Dopico.
Large conductance, voltage- and calcium-gated potassium (BK) channels are known to cluster in cholesterol-rich cell membrane domains. Furthermore, cholesterol-BK channel interaction usually results in reduced channel activity (Po), as reported with native (Bolotina et al., 1989) and recombinant (Crowley et al., 2003) channels. We previously communicated that cholesterol-inhibition of BK channels cloned from rat cerebral artery myocytes (cbv1; AY330293) and reconstituted into POPE:POPS (3:1) bilayers displayed enantio-specificity and stereoselectivity, strongly suggesting that the decrease in Po involved cholesterol-recognition by a protein surface (Bukiya et al., Biophys. Soc. 2010). Using a similar system, we now demonstrate that cholesterol (16-33 mol%) inhibition of cbv1 channels is similar whether the channel is open by positive voltage or increased intracellular calcium, suggesting that the cholesterol-protein(s) interaction leads to altered channel function independently of the signal that gates the channel. Because the channel phenotype was characteristic of homomeric slo1, we hypothesize that regulation of BK gating by cholesterol involves an interaction between the steroid and the cbv1 subunit. One of the motifs that favors cholesterol-protein interactions is CRAC: -L/V-(X)(1-5)-Y-(X)(1-5)-R/K-, where X denotes any residue (Epand, 2008). We used CRAC sequence patterning and found ten CRAC motifs in cbv1, three in the core (S0-S6) and seven in the C-tail (S7-C end) domain. Thus, we next evaluated the cholesterol sensitivity of cbv1 channels truncated immediately after S6 (trS6cbv1). Remarkably, trS6cbv1 was consistently resistant to cholesterol-induced modulation (n=11) under conditions where *wt* cbv1 remained sensitive. Therefore, the C-tail domain confers cholesterol-sensitivity to cbv1 channels. We are currently using a combination of computational dynamics, sequential cbv1 truncation, and point mutagenesis in CRAC motifs to determine the relative contribution, if any, of these motifs to the cholesterol-sensitivity of BK channels.
Support: HL104631 (AMD), UTHSC Neurosci. Postdoc. Fellowship (AKS).

3159-Pos Board B264
A Functional Analysis of NaK at the Single Channel Level
Raymond W. Bourdeau, Valeria Vasquez, Julio F. Cordero-Morales, Eduardo Perozo.
NaK is a non-selective monovalent cation channel from *Bacillus cereus*. Despite being unable to discriminate between Na^+ and K^+, NaK shows high sequence similarity to other K^+ channels. Based on recently solved crystal structures in the closed and putatively open state, NaK exhibits an overall architecture similar to that found in the pore domain of tetrameric K^+ channels. Rb^+ influx studies suggest the channel conducts cations, however net flux is unusually low for a channel. The absence of electrophysiological data from NaK precludes significant understanding of it's functional behavior. Using a random mutagenesis approach together with a K^+ transport based screen, we have identified gain-of-function mutants in an attempt to develop a system for electrophysiological studies. One of these purified and reconstituted mutants was further studied by liposome patch-clamp. The channel displays non-selective conductances at 25 and 91 pS and is characterized by a low probability spiking

behavior. In addition, we show that NaK undergoes a voltage-dependent inactivation process, which is functionally similar to that seen in K^+ channels. This inactivation may contribute to the low flux of Rb^+ through NaK. Our functional characterization, along with the known crystal structures, now allows us to use NaK as a model system to further investigate structure-function correlations in non-selective channels and related selectivity filters.

3160-Pos Board B265

Engineering the hERG1 Selectivity Filter into the NaK Pore Domain

Julio F. Cordero-Morales, Vishwanath Jogini, Valeria Vasquez, Raymond W. Bourdeau, Haibo Yu, Benoit Roux, Martin Tristani-Firouzi, Eduardo Perozo.

Comparison of the hERG1 selectivity filter sequence and pore helix with a variety of prokaryotic ion channels revealed an unusually high sequence identity (63%) with the non-selective cation channel NaK (~19 amino acids). Taking advantage of this remarkable similarity, we have used NaK as a template to carry out a structural analysis of a hERG1-like NaK selectivity filter in K+. To engineer the NaK filter into that of hERG1, we substituted two critical residues, V59S in the pore helix and D66F at the selectivity filter (equivalent to positions Ser620 and Phe627 in hERG1, respectively). The final construct shares 73% sequence identity with the hERG1 selectivity filter, equivalent to the closest orthologue of hERG1, the bovine ether-a-go-go channel (bEAG1). Crystals of this engineered hERG-NaK diffracted to 2.8 Å resolution and were solved by molecular replacement using closed NaK as template. The crystal structure of hERG1-like NaK in 100 mM K+ revealed a striking similarity to the all canonical K+ channel filters in the conductive conformation (RMSD= 0.5 Å with the KcsA filter) and shows three major differences compared to WT NaK. First, hERG1-like NaK filter revealed four equivalent K+ binding sites. Second, the side chain of Phe66 residue establishes critical packing interactions with the adjacent aromatic residues from the pore helix. Third, hERG1-like NaK shows hydrogen bond interactions through a water molecule behind the selectivity filter, which is absent in WT NaK, but present in KcsA. We suggest that the hERG-NaK construct represents a unique tool to investigate the properties of the hERG1 channel selectivity filter at atomic level.

Muscle: Fiber and Molecular Mechanics & Structure II

3161-Pos Board B266

Radial Motion of Myosin Heads in Isolated Intact Rat Myocardium in Diastole

Gerrie P. Farman, HsiaoMan Hsu, David Gore, Edward J. Allen, Kelly Q. Schoenfelt, Younss Ait Mou, Thomas C. Irving, **Pieter P. de Tombe**.

The main cellular mechanism that underlies the so-called ''Frank -Starling Law of the Heart'' is an increase in the responsiveness of cardiac myofilaments to activating Ca^{2+} ions at longer sarcomere lengths (SL). The fundamental mechanism responsible for this increase in responsiveness has been elusive, despite considerable experimental scrutiny. Here we tested the hypothesis that the increase in calcium sensitivity upon increasing SL is correlated with a radially outward movement of the myosin heads during diastole. 2D x-ray diffraction patterns were obtained from electrically stimulated intact, twitching papillary muscle isolated from rat hearts during a 10 ms time window in diastole just prior to electrical stimulation. A range of sarcomere lengths was compared either at L_{max} (SL= ~2.3 μm) or following a quick release to slack length (SL=~1.9μm). The relative position of myosin heads was first assessed by the I_{11}/I_{10} equatorial intensity ratio. To our surprise, I_{11}/I_{10} was negatively correlated with SL, i.e. I_{11}/I_{10} was less at L_{max} vs. slack length. A more direct measure of the radial position of the myosin heads can be estimated from the position of the first maxima on the unsampled myosin layer lines, which are prominent in diastole. The intensity maxima, when examined pair-wise, moved outwards to a maximum of 5-6% for a 0.4μm change in SL indicating that the heads must be moving radially outward at slack length. Our data suggest that myofilament length dependent activation does not derive from a radial extension of the myosin heads at the long SL and must, therefore, involve some other mechanism. Supported by NIH HL75494 and RR08630.

3162-Pos Board B267

Electron Tomography of Thick Sections of Insect Flight Muscle

Claudia L. Vargas, Anthony Warrington, Kenneth A. Taylor, Susan Hester, R.J. Perz-Edwards, Michael K. Reedy.

Insect flight muscle (IFM) is a good model system within which to visualize actin-myosin interactions due to its highly ordered lattice of actin and myosin filaments. Lethocerus flight muscle is perhaps the best ordered muscle in nature. Electron tomography (ET) of Lethocerus IFM has recently resulted in a model for the weak to strong transition that incorporates large azimuthal changes in the position of the lever arm compared to that predicted from crystal structures of myosin subfragment 1 in both the nucleotide free and transition states (Wu et al. PLoS-ONE, Sept. 2010). Those studies did not visualize the S2 domain in either the raw tomogram or in subvolume averages which would clarify the crossbridge origin. Here we have used ET of IFM fibers in rigor in which the filament lattice has been swollen in low ionic strength buffer to view where S2 emerges from the thick filament backbone as a test of the weak to strong transition. Previous ET on myac layers (single filament layers containing alternating myosin and actin filaments) of these same swollen rigor fibers revealed the S2 domain with clarity. In the present work, we are examining 80 nm thick transverse and longitudinal sections of swollen rigor IFM fibers in order to visualize all of the crossbridges originating from each 14.5 nm crown on the thick filament, but especially the so-called lead bridges, which bind the thin filament within the same target zone of isometric contraction. Class averages of both thick filaments as well as myac layers are being pursued. The thick filaments show subfilaments in the backbone and many of the myac layer raw repeat subvolumes show S2. Progress on this study will be presented. Supported by NIGMS and NIAMSD.

3163-Pos Board B268

Vanadate Responses of Insect Fight Muscle

Robert J. Perz-Edswards, Rebecca L. Porter, **Michael K. Reedy**.

When exposed to sodium orthovanadate (Vi), permeabilized insect flight muscle (IFM) from *Lethocerus* water bugs behaves differently in three ways from vertebrate skeletal muscle (VSkM) under similar conditions.

Weak binding of Vi in RLX: IFM treated 5-10 min with 250 μM Vi in Lo-μ RLX buffer (= Vi-RLX) (pCa ~9.0; μ ~90 mM), then washed free of Vi in Lo-μ RLX, and placed in Lo-μ ACT buffer (pCa ~4.5; μ ~105 mM) rises to maximal isometric tension far more slowly (fives of minutes) than untreated IFM, suggesting a weak-binding, slowly reversible interaction with (presumably) myosin.

IFM 100x more sensitive to Vi than VSkM: Exposing maximally Ca-activated IFM to 250 mM Vi in Lo-μ ACT (= Vi-ACT) quickly suppresses active-state tension 97-100% and induces relaxed-state X-ray diffraction structure. Vi-ACT-exposed IFM recovers little or no active-state tension during 30-60 subsequent minutes in Lo-μ ACT. Vi-trapping in IFM is so strong that just 2 μM Vi ultimately (30-50 min) suppresses active tension in Lo-μ ACT by ~75%, suggesting that 50% IFM force inhibition would require 1 μM or less Vi, versus 45-94 μM in VSkM.

Only crossbridges opposite target zones are Vi-trapped: Despite 97-100% paralysis of Ca-activated isometric force production by Vi-ACT, Vi trapping only affects myosin crossbridges opposite actin target zones. When stretched 2-4%, fibers recover active tension capability. Post-Vi washout with RLX, followed by 2-4% stretch, followed by Lo-μ ACT exposure, generates significant Ca-activated tension, because at the longer sarcomere length target zones have moved toward myosin heads that at rest length were unable to reach the 2/7 fraction of IFM actin monomers (Wu *etal*, PLoS One **5**: e12643 (2010)) that accept strong-binding crossbridges, ATPase cycling and tight Vi trapping. Slow 3% length-cycling in Vi-ACT Vi-traps **all** accessible IFM crossbridges. (Support: NIH, MDA).

3164-Pos Board B269

N-Benzyl-P-Toluenesulfonamide (BTS) Traps the Myosin Head in a Conformation Associated with Strong Myosin Based Layer Lines and Weak, Rapidly Reversible Actin Binding even in the Absence of Nucleotide

Theresia Kraft, Ante Radocaj, **Bernhard Brenner**.

During its working cycle myosin proceeds through several structural changes coupled to changes in the state of the nucleotide. Details of the coupling between nucleotide state and myosin conformation are still unclear. Several lines of evidence suggest that for the same nucleotide state several conformations of the myosin head coexist while the state of the nucleotide may only determine their relative proportions (e.g., Xu et al., Biochemistry 2003; Nesmelov et al., Biophys J. 2008).

We were particularly interested whether with ADP or without nucleotide, i.e., when myosin binds tightly to actin, we can find experimental interventions for which (1) strong myosin based layer lines (MLLs) can be seen in 2D-X-ray diffraction patterns as sign of the closed, pre-power stroke conformation, and (2) binding kinetics of myosin to actin can be determined by fiber stiffness. While we were unsuccessful with blebbistatin, we found N-Benzyl-p-toluenesulfonamide (BTS) to accumulate myosin heads, both without nucleotide and with ADP, in structural states that generate strong MLLs. While BTS had no effect on MLLs in the presence of ATP, with ADP or no nucleotide BTS generated MLLs as strong as seen with ATP at high temperature. This suggests that